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Comparison of Free Radical Scavenging Capacities of Methanol Extract of Two Mint Species by Fixed Point and Kinetic Methods

Shadia Sirry [1,]* and Hind Babiker[1, 2]

[1]*Chemistry Department, Faculty of Science, Taibah University, P.O. Box 30002, Saudi Arabia*

[2] *Biochemistry and Molecular Genetic Department, Faculty of Science, Al-Neelain University, Sudan*

**Author to whom correspondence should be addressed; smrry90@hotmail.com.edu.sa*
Tel.: +966502008141

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Abstract: Mentha Longifolia (ML) and Mentha Puleguim (MP) are usually used as a flavor and medicinal plants in Saudi Arabia. The present study was carried out to compare the potential of methanol extract of fresh leaves of ML and MP as free radical (DPPH) scavenger by fixed point and kinetic methods. In a fixed point method, free radical scavenging capacity was expressed by effective concentration, EC_{50}. However, in kinetic method, capacity expressed by rate constant value. Some other antioxidants parameters like total flavonoids content and total phenolic content were also estimated. Flavonoid content was obtained as catechin equivalent and total phenolic content as gallic acid equivalent. The obtained results indicate that methanol extract of MP possess a high concentration of flavonoids (6.55± 0.42 mg/100 ml) and of phenolic (3.72 ±0.29 mg/100 ml) compared to that of ML flavonoids (3.22± 0.43 mg/100 ml) and of phenolic (1.91 ±0.06 mg/100 ml).

Keywords: *Free radical scavenging capacity; Antioxidant, DPPH, Kinetic; Total phenolic content; Total flavonoids*

I. Introduction

Free radicals and other reactive species (oxidants) are produced in the body as a by-product of aerobic metabolism or as a result of environmental stress. It is characterized by the presence of unusually high concentrations of reactive species like superoxide, hydroxyl alkoxyl, hydroperoxyl, nitric oxide and nitrogen dioxide radicals [1]. Excessive in vivo production of these radicals induce oxidative damage in protein, lipids, and DNA, which leads to chain reaction and oxidative stress.

Dietary sources of antioxidants are secondary metabolites from plants, which are react with free radicals to terminate the chain reaction and prevent the oxidative stress. However, these secondary metabolites are not essential for basic growth and development of plants, but they are synthesized mainly to face biotic and abiotic stresses. Usually secondary metabolites that have potential effects as antioxidants include phenolic and flavonoid compounds.

The free radical scavenging capacity has been measured by several methods [2] among those DPPH (2,2-Diphenyil-picrylhydrazyl) assay is considered as a simple method to evaluate the antioxidant activity of both pure compound and plant extracts [3]. The free radical $DPPH^{\bullet}$ is a stable free radical with deep violet color and easily measured by UV-Vis spectrometry at 515 nm. When

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DPPH• react with antioxidant, the deep violet color was bleached and the percentage of inhibition of color is determined as:

$$\%I = 100\frac{[DPPH]}{[DPPH]_o} \quad (1)$$

[DPPH] $_o$: is the initial concentration, [DPPH] is the concentration of DPPH after reacting with antioxidant. DPPH assay usually carry out at fixed time and variable concentrations of antioxidant. Antioxidants capacity was measured as $IC_{50,}$ which means concentration of antioxidants necessary to inhibit DPPH concentration by 50%. DPPH assay give an information about antioxidants capacity at definite time. Some researchers reported IC_{50} after 15 minutes incubation and others after 30 minutes [4]. However, the estimations of IC_{50} at fixed-point time does not take into consideration the effect of kinetic parameter [5]. Plant extracts may contain more than one type of antioxidants, which react with DPPH radical, some antioxidants are fast and others are slow. In order to overcome this problem some workers suggested that kinetic parameters is an important to determine the actual capacity of antioxidants [6 -9]. The kinetic of the reaction between antioxidants and DPPH radical were reported as first order and other as second order reaction [9].

The Genus Mentha, belonging to the family Lamiaceae, contains many species. Mentha species are usually utilized as herbal tea in medicine, taste and aroma, folk remedy, raw material for pharmaceutical, cosmetic, perfume and as food industry [10]. Many studies on antioxidant activity of mint species had been performed and revised that the activity is mainly due to phenolic components, such as flavonoids [11-12], phenolic acids and phenolic terpenes [13]. Mentha Longifolia (ML) and Mentha Pulegum (MP), are usually utilized as flavoring in Saudi Arabia and their local names are Al-Madina hasawy mint and Mugrabi mint, respectively [14-15]. The aim of this study is to compare the free radical (DPPH) scavenging capacities of fresh leaves methanol extract of MP and ML by fixed point and kinetic methods. Total phenolic content and total flavonoids were also determined to support the study.

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II. Experimental section

II.1. Apparatus:

A grinding machine, a shaker device (GFL), reduced pressure rotatory evaporator, (Buchi R 210), UV/Visible spectrophotometer (CINTRA 6 GBC)

II.2. Materials

DPPH, gallic acid and absolute methanol were from Sigma Aldrich (USA). Folin–Ciocalteu phenol reagent was obtained from Merck (Darmstadt, Germany). Sodium carbonate, sodium nitrite and aluminum was purchased from chemical pure company.

II.3. Preparation mint extracts

Fresh leaves of mint species were purchased from local market, washed and grounded. 2 g of the grounded mints were shaken with 100 ml methanol in mechanical shaker at 200 rpm for 2 hours. The extracts were kept at 5˚C for 24 hours and evaporated under reduced pressure rotatory evaporator, (Buchi R 210)

II.4. Free radical scavenging capacity

The ability of free radical scavenging of mint species was assayed by 1,1-Diphenyl-2-picrylhydrazyl radicals (DPPH) by adding constant concentration of DPPH to different concentrations of mint extracts and measuring the degradation of color intensity of DPPH 3 (Brand-Wiliam et al.,

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1995) at wavelength of maximum absorbance, λ_{max}.equal 516nm by UV/Visible spectrophotometer (CINTRA 6 GBC)

II.5. Kinetic of free radical scavenging

Kinetic of free radical scavenging ability of mint extracts was determined by mixing of DPPH (0.18mmole) and mint extracts (0.0634 mg/mL) and measuring the absorbance at wavelength 516nm at time interval 0-120 minutes. Remaining DPPH concentration was calculated from calibration curve of DPPH. kinetic studies were investigated three times at room temperature.

II.6. Determination of Total Flavonoids

In 10 mL volumetric flask, 4mL of water, 2mL of mint extracts and 0.3 mL of $NaNO_2$ (5%) were mixed. After 5min 3mL of $AlCl_3$ (10%) was added then after 6min 1mL of NaOH (4%) was added. The mixture was completed to 10mL by water and the absorbance was measured at 510 nm against blank with spectrophotometer. Catechin was used as standard and the calibration curve was obtained by the same manner of extract utilizing 0.2-1mL catechin solution (500µg/mL) [16-17].

II.7. Determination of total phenolic compound

Total phenolic contents in mint extracts were quantified by Folin–Ciocalteu reagent [18] as Gallic acid equivalent GAE.

III. Results and Discussion

Methanol is polar solvent commonly used as extraction of flavonoids and other phenolic compounds. In this study, it was utilized as extractant for fresh ML, MP mint leaves (Fig.1) and the extract yields were calculated and given in table 1. It is clear that the yield of ML extract is slightly higher than MP.

Figure 1*: Mentha longifolia (ML) and Mentha pulegium (MP) leaves*

Table 1: *Percent of yield of ML and MP extract*

type of mint	Weight,g	%yeild
ML	0.0862	4.31
MP	0.0792	3.96

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III.1. Free Radical Scavenging Capacity (DPPH assay):
III.1.1.Fixed point method

Radical scavenging ability is usually evaluated by the ability of antioxidant to scavenge DPPH$^{\bullet}$ radical. The violet color of DPPH$^{\bullet}$ is due to the transferring of free electron around its molecule. After reacting with antioxidant, it is change to yellow due to scavenging of free electron. Free radical scavenging ability are deduced spectrophotometrically by following the color inhiption of DPPH$^{\bullet}$ at mximum absorbance wavelength (λmax = 517 nm).The percent of inhiption of DPPH$^{\bullet}$, %I, is given as:

$$\%I = \frac{(A_{original} - A_{final})}{A_{original}} \quad (2)$$

% I depend on concentration and time [3,19].The values of % I of constant concentration of DPPH (0.08M) as a function of concentration of mint extract at constant time (15minutes) were shown at Fig 2. The amount of free radical inhibnition increased as the concentration of mint extract increased. The concentration of antioxidant that scavenge 50% of original DPPH$^{\bullet}$ radical called effective concentration (EC_{50}) and its characteristic to free radical scavenging ability for antioxidants. The lower value of EC_{50},the larger of scavenging capacity. EC_{50} values of ML and MP extracts are given at table 2 and compared with effective concentrations of some standards which were reported previously.

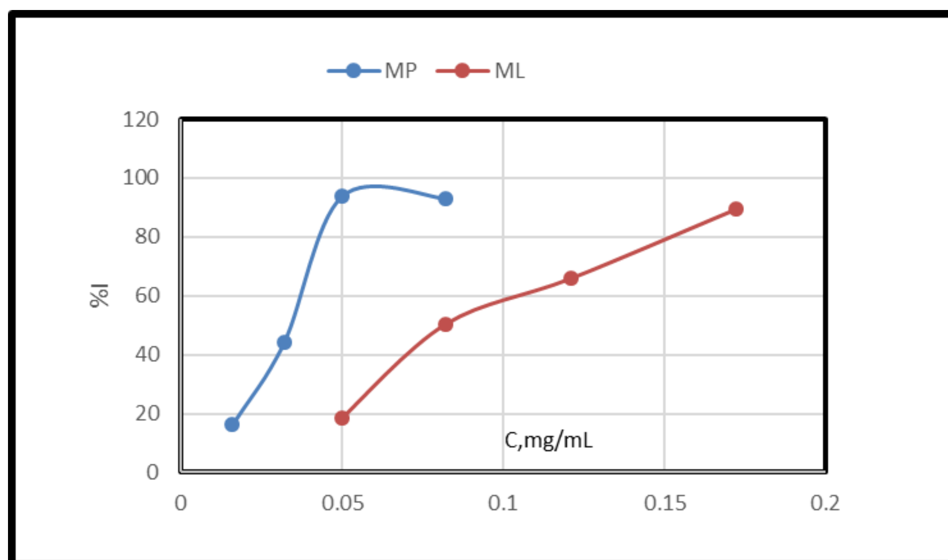

Figure 2: *Effect of effective concentration for DPPH radical scavenging*

Table 2*: Comparison of Efficient concentration of ML, MP extract with previous studies of ML, MP and standards*

Type of mint	Ec_{50},mg/ml	Reference
ML	0.085	Present study
MP	0.034	Present study
ML	0.057	[20]
Mentha spicata L	0.021	[21]
ML	0.086	[22]
Gallic acid	0.001	[23]
Catechin	0.007	[24]

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The lower value of EC_{50} of MP extract (0.034mg/mL) with respect to ML extract (0.085mg/mL) prove that MP extract is effective as radical scavenging than ML. The values of IC_{50} of ML in the present study was cocordant with ML of Özgen *et al.* [22].

III.1.2. Kinetic method

The reaction between antioxidants and free radical is time dependent and reached a steady state after a period [9].Thus, reaction kinetics between antioxidant and DPPH radical give more accurate results about the impact of antioxidants than fixed-point antioxidant capacity.

To study rate of free radical scavenging of two mint methanol extracts, a constant concentration of mint extracts (0.0634mg/mL) and DPPH (0.182mmole/L) were mixed and the reaction was followed spectrophotometrically at λ_{max} 517 nm from 0 minute to 150 minutes (Fig.3)

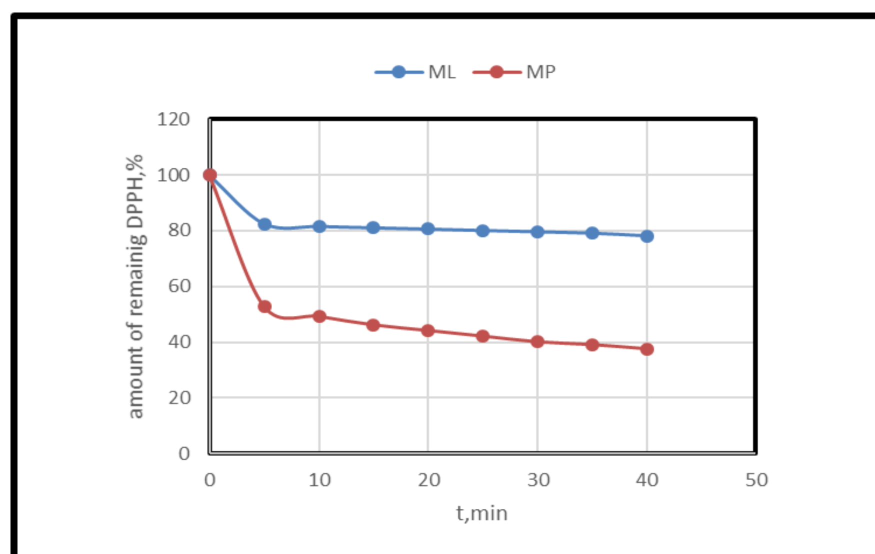

Figure 3: *Rate of free radical scavenging of ML and MP mint extracts*

From figure, it was obvious that the rate of both extracts as free radical scavenging have two stages initial fast then slow. The reaction of antioxidant in mint and DPPH radical may occur according to two independent parallel mechanism: Hydrogen atom transfer, HAT (fast stage) and sequential proton loss electron transfer, SPLET, (slow stage) [5,7]. However according to the time of reaching to steady state, the kinetic reaction between antioxidants and DPPH has been classified as fast (<5 min), intermediate (5-30 min) and slow (>30 min [3,19]. The steady state of ML and MP were >120 min therefore, the reaction between mint extract antioxidants and DPPH is classified as slow. The rate of MP extract as free radical scavenging more than rate of ML extract. Order of the reaction between DPPH and mint extracts gives an information about the rate constants of the reaction and number of active molecules take part. The scavenging kinetic of DPPH radicals by antioxidants may follow second order or pseudo first order according to the ratio of amount of antioxidant: DPPH [24-25].The order of the reaction are obtained by fitting the experimental data to both first and second order kinetic equations.

The reaction between DPPH radical and antioxidants in mint extracts is represented by:

$$A + DPPH \rightarrow P \quad (3)$$

A: Antioxidant in mint extracts P: product

This reaction may fit either first or/and second order kinetic models:

Frist order kinetic model:

$$\frac{-d[DPPH]}{dt} = k_1[DPPH] \quad (4)$$

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Second order kinetic model:

$$\frac{-d[DPPH]}{dt} = k_2[DPPH][A] \tag{5}$$

Assuming the presence of same amount of A and DPPH

$$\frac{-d[DPPH]}{dt} = k_2[DPPH]^2 \tag{6}$$

Integration form of the equations 4 and 6 are

$$\ln[DPPH] = \ln[DPPH]_0 - kt \tag{7}$$

$$\frac{1}{[DPPH]} - \frac{1}{[DPPH]_o} = K_2 \tag{8}$$

K_1 and K_2 are the rate constants for first and second order, respectively
$[DPPH]_o$ is the original concentration of DPPH and [DPPH] is the concentration at time t and determined from the calibration curve of DPPH, Fig. 4

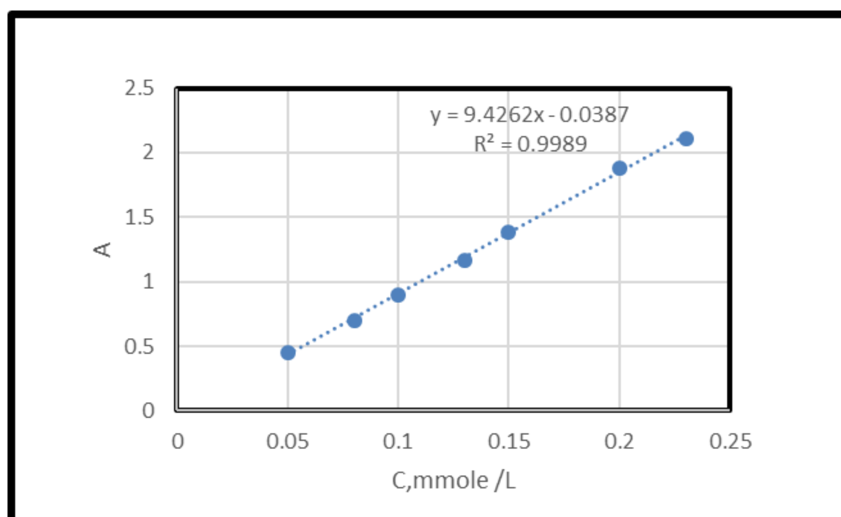

Figure 4: *Calibration Curve of DPPH*

Equation 7 and 8 were applied to explore the fitness of $DPPH^{\bullet}$ scavenging by ML and MP mint extracts to first or second order (Fig.5 and 6).

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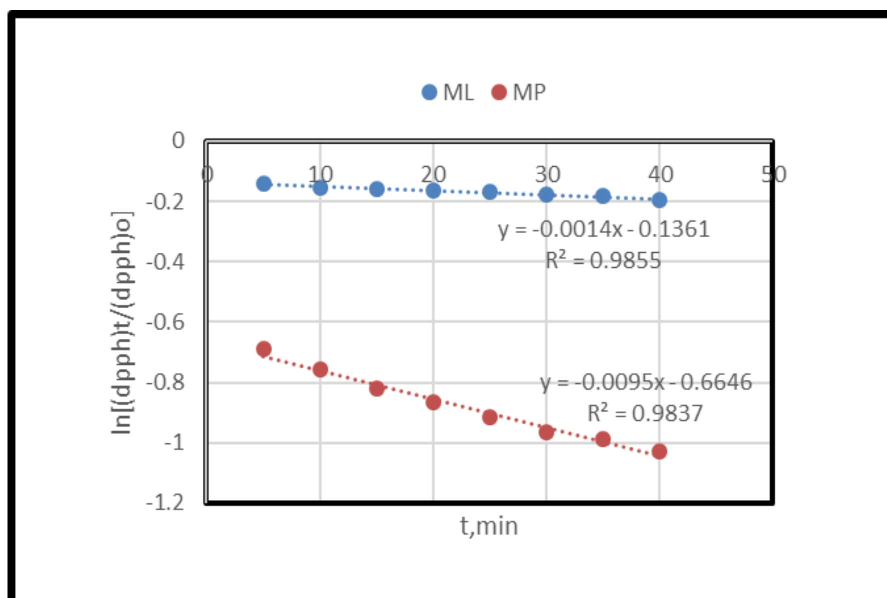

Figure 5: *First order kinetic equation of free radical scavenging of ML and MP mint extracts*

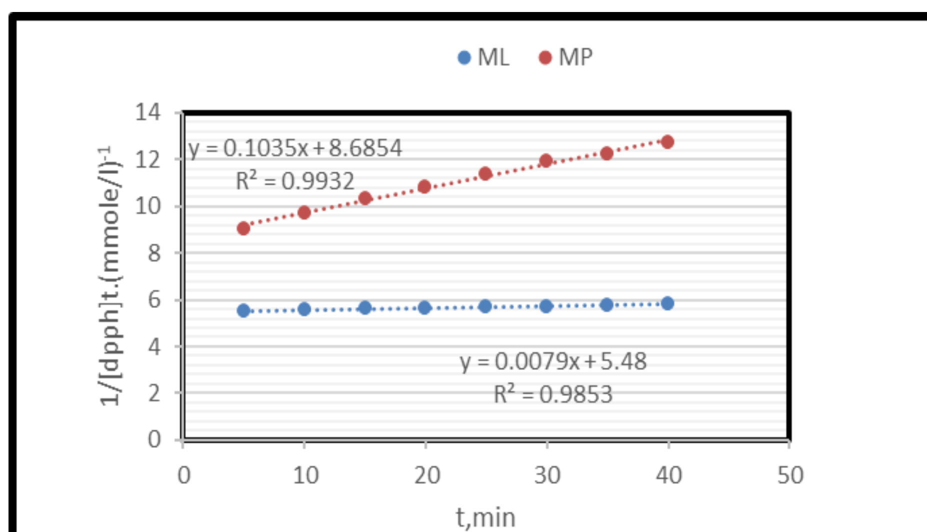

Figure 6: *Second order kinetic equation of free radical scavenging of ML and MP mint extracts*

From the results it was investigated that both first and second order kinetic equations were good fitted with linear regression constant R^2 more than 98%. The rate constants for first and second order equations ($K_1$and K_2) are obtained from the slopes data and given at table (6). The applicability of both first and second order reaction may be due to the presence of two groups of antioxidants, fast and slow antioxidants. A previous studies [26-27] reveals that various mentha species contain antioxidants like phenolic (rosmarinic acid, caffeic and luteolin derivatives) as major antioxidants and ascorbic and carotenoids as minor antioxidants. According to Brand William *et al.*, [3, 28] ascorbic acid is a rapid antioxidant, rosmarinic acid is classified as intermediate antioxidant and caffeic acid compounds as slow antioxidants. The overall rate of interaction between the same amounts of plant extracts and DPPH were used as a measure of antioxidants capacity of mint species. The rate constant values, k_1 and k_2, of MP extract more than ML, thereby, the antioxidants ability of methanol extracts of MP more than ML mint.

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Table 3: *Kinetic Constants of ML and MP*

	ML	MP
k_1 min^{-1}	0.0014	0.0095
R^2	0.986	0.984
k_2 $(mg/ml)^{-1}min^{-1}$	0.0079	0.1035
R^2	0.985	0.993

III.2. Antioxidant characteristics

The antioxidant characteristics of methanolic extraction of fresh MP and ML leaves was achieved by total flavonoids and total phenolic.

III.2.1. Determination of Total Flavonoid Content

Flavonoids are considered as antioxidants by neutralizing hydroxyl and superoxide radicals and by chelation. Several investigator have established that Mentha species contains wide range of flavonoids [29-31]. Flavonoids in mint extracts was determined according to catechin content The calibration curve of catechin was performed and given in Fig.7. Total flavonoids of methanolic extracts of ML and MP were determined spectrophotometrically as standard catechin content (CE) in mg/100mL extract (Table 4)

MP extract have higher flavonoid content than ML extract. The same trend was observed in a previous work [32] and opposite observation for the result was reported by [33].

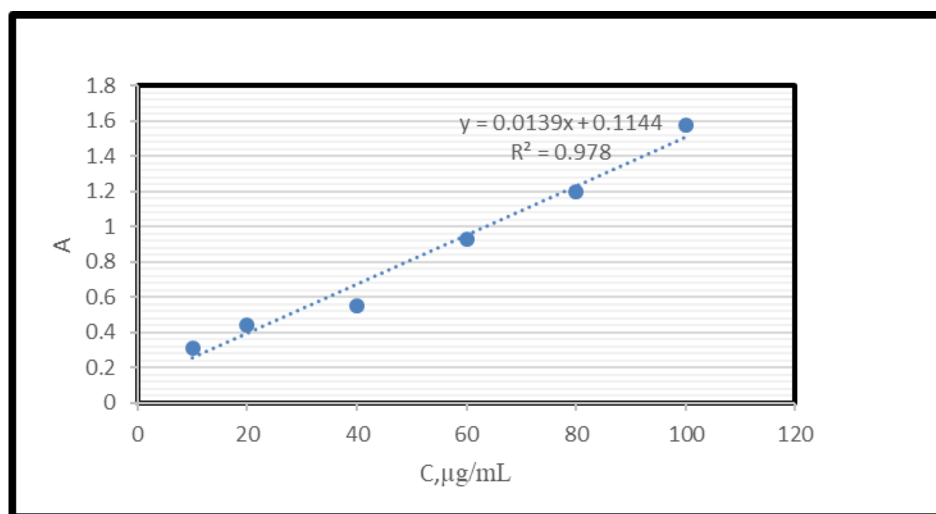

Figure 7: *Standard curve of catechin*

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***Table 4**: Flavonoids content of methanolic extract ML and MP as catechin equivalent CE (mg/100mL)*

type of mint	A	CE, mg/100ml extract	Average (standard deviation)
	0.293	3.212	3.2242±0.432
ML	0.270	2.799	
	0.318	3.662	
	0.454	6.107	6.557±0.426
MP	0.501	6.953	
	0.482	6.612	

III.2.2. Determination of total phenolic content, TPC

Phenolic compounds are the most important compounds in mint species as account of their relation as antioxidants. Total phenolic content was estimated spectrophotometrically as gallic acid equivalent (GAE) by Folin reagent at 760 nm. (Fig.8), and Table 5.Total phenolic content in MP is more than that of ML. Similar trend was obtained in previous study of same type of Mentha species [34].

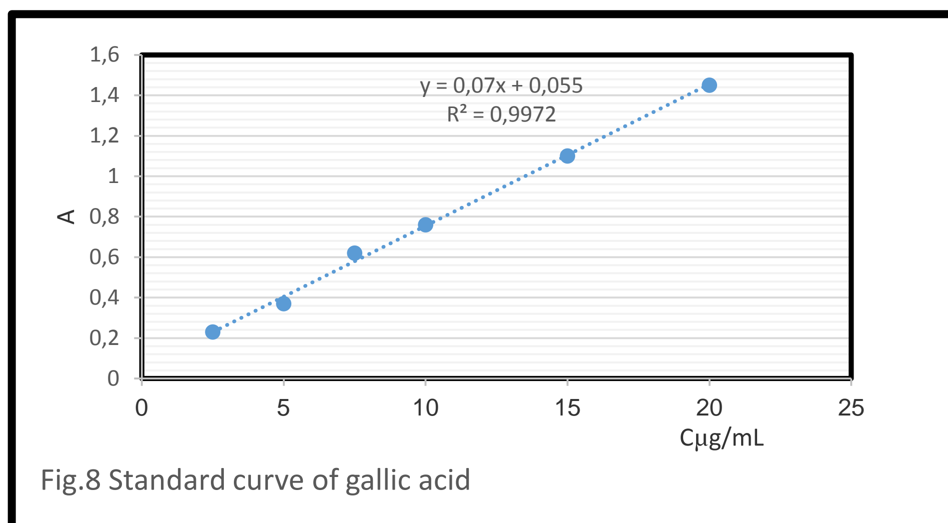

***Figure 7:** Standard curve of Gallic acid*

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Table 5: *Determination of total phenolic as miligram gallic acid per 100 mL extract (µg GAE/ml).*

type of mint	A	TPC, mg/100ml extract	Average (standard deviation)
	0.194	1.986	1.914±0.062
ML	0.187	1.886	
	0.186	1.871	
	0.197	4.057	3.724±0.292
MP	0.181	3.600	
	0.178	3.514	

IV. Conclusion

This study is a comparative study of free radical scavenging capacity of mentha Longifolia, ML and Mentha Puleguim extract by fixed point and kinetic method. In Both methods, the free radical scavenging capacity of methanol extract of MP was found to be higher than that of ML.MP extract also exhibited a higher concentration of total phenol, and total flavonoids than that of ML extract.

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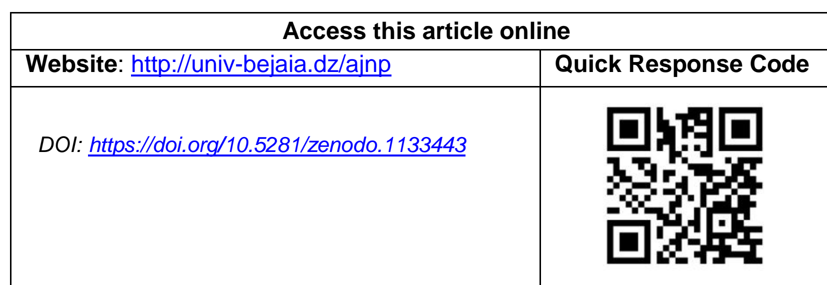

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